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Prostaglandin E2 activates Stat3 in neonatal rat ventricular cardiomyocytes: A role in cardiac hypertrophy

Miguel A. Frias *, Michela C. Rebsamen, Christine Gerber-Wicht, Ursula Lang

Division of Endocrinology, Diabetology and Nutrition, University Hospital, 24, rue Micheli-du-Crest, CH-1211 Geneva 14, Switzerland

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Abstract

Objective: The purpose of this study was to investigate whether prostaglandin E2 (PGE_2) induces Signal transducer and activator of transcription 3 (Stat3) activation in neonatal rat ventricular cardiomyocytes and if so to determine the possible role of this activation in PGE_2-induced hypertrophic responses.
Methods: Stat3 activation and its nuclear phosphorylation were determined by electrophoretic mobility shift assay (EMSA) and by Western blots, respectively. Protein synthesis was assessed by [^{3}H]-leucine incorporation into total protein and cell surface was quantified by microscopic analysis.
Results: We found that PGE_2 induces a concentration- (1–100 nM) and time-dependent increase in Stat3 activation, reaching maximal values after 90 min of stimulation. Experiments with agonists and antagonists of the PGE_2 receptor subtypes EP1–EP4 indicate that PGE_2 activates Stat3 mainly through the EP4 receptor. We further observed that the extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor U0126 abolishes PGE_2-induced Stat3 activation whereas the p38 MAP kinase blocker SB203580 has no effect. Nuclear Stat3 phosphorylation induced by PGE_2 is also suppressed by the translation and transcription inhibitors, cycloheximide and actinomycin D, respectively. Transfecting ventricular cardiomyocytes with a small interfering RNA (siRNA) targeting rat Stat3, we obtained an approximately 70% reduction in Stat3 expression, 24 and 48 h after electroporation. In these Stat3-silenced cells, the PGE_2-induced increase in protein synthesis and cell surface is strongly inhibited.
Conclusion: In ventricular cardiomyocytes, PGE_2 induces the activation of Stat3 which plays an essential role in PGE_2-induced increase in cell size and protein synthesis. The activation of Stat3 occurs mainly through EP4 and involves ERK1/2 as well as newly synthesized protein(s).
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Keywords: Hypertrophy; Prostaglandins; Signal transduction

This article is referred to in the Editorial by Schaub and Hefti (pages 3–5) in this issue.

1. Introduction

Prostaglandin E2 (PGE_2) is involved in multiple physiological and pathological processes such as vasodilation, proliferation and inflammation. In the heart, PGE_2 has been shown to be secreted under stress conditions including myocardial infarction [1]. We have previously shown that PGE_2 is released in ventricular cardiomyocytes following stimulation with aldosterone [2] which has been reported to induce ventricular hypertrophy in rats [3]. Similarly, increased cardiac PGE_2 production was reported in an animal model of left ventricular hypertrophy [4]. Recently, PGE_2 was found to induce hypertrophic responses such as an increase in cell surface and protein synthesis as well as an activation of ANP and BNP promotors in ventricular cardiomyocytes through the PGE_2 receptor subtype EP4 [5,6].

The development of cardiac hypertrophy is a complex process involving a variety of signaling pathways. A number

* Corresponding author. Tel.: +41 22 3729322; fax: +41 22 3729330.
E-mail address: Miguel.Frias@medecine.unige.ch (M.A. Frias).

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of studies have shown that the Signal transducer and activator of transcription 3 (Stat3) plays a major role in the development of cardiac hypertrophy [7–9]. Indeed, cardiac specific overexpression of the Stat3 gene in transgenic mice results in myocardial hypertrophy with an increased expression of ANP and β-MHC [7]. Stat3 is activated under stress conditions such as pressure overload and acute myocardial infarction as well as by the hypertrophic hormone angiotensin II [10–13]. Moreover, Stat3 promotes cardiomyocyte survival and hypertrophy in response to various pathophysiological stimuli [14–16].

As reported in several studies, the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway is another signaling cascade playing an important role in the development of cardiac hypertrophy. Indeed, multiple *in vivo* and *in vitro* studies, using pharmacological and genetic approaches, showed that the MEK1/2–ERK1/2 signaling cascade regulates important aspects of the hypertrophic response in cardiomyocytes [17–19].

At the present time there is no information concerning the effects of PGE_2 on Stat3 in cardiac cells. In this study we investigated the mechanism of action of PGE_2-induced Stat3 activation involving ERK1/2 as well as the role of Stat3 in PGE_2-stimulated hypertrophic responses in neonatal rat ventricular cardiomyocytes.

2. Material and methods

2.1. Cell culture

This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1966). Neonatal cardiac cells were isolated from 1 to 2-day-old Wistar rats ventricles by digestion with trypsin-EDTA and type 2 collagenase as previously described [20]. Briefly, once the sequential digestions were terminated, the cells were pooled in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Groningen, the Netherlands) supplemented with 10% fetal calf serum (FCS, Biochrom AG, Berlin, Germany), penicillin (100 units/ml) and streptomycin (10 µg/ml) and seeded in 150 cm^2 flasks to allow selective adhesion of cardiac fibroblasts [21]. Thereafter, cardiomyocytes were decanted from the plates and seeded in petri dishes (60 or 90 mm, Costar, Cambridge, MA). The majority of cultured cardiomyocytes began to contract spontaneously within 24–48 h of plating (30–50 beats/min). For all experiments described herein, cells were used in the third day of culture, after 16–20 h in DMEM medium depleted in FCS.

2.2. Western blotting

Cardiomyocytes were starved of serum overnight and subjected to treatment as indicated in the figure legends. After stimulation in serum-free DMEM, cells were washed with ice-cold PBS and lysed with 50 µl of the following buffer: Tris-HCl (50 mM, pH 7.4), NaCl (150 mM), glycerol (10%), EDTA (2 mM), EGTA (2 mM), Triton X-100 (1%), β-glycerophosphate (40 mM), NaF (50 mM) containing a cocktail of protease inhibitors (Roche, Mannheim, Germany). In order to inhibit serine/threonine and tyrosine dephosphorylation, we inhibited serine/threonine and tyrosine phosphatases by okadaic acid (100 nM) and sodium orthovanadate (0.2 mM) respectively.

Nuclear or total cell proteins (10–30 µg) were separated by SDS-PAGE on an 8 or 12% acrylamide gel and blotted onto nitrocellulose membrane. Afterwards, membranes were incubated overnight at 4 °C with specific polyclonal antibodies. The blots were then washed and incubated for 1 h with a horseradish peroxidase-labeled anti-rabbit antibody (Covalab, Oullins, France). Specific bands were visualized with a chemiluminescence kit (Amersham, Zürich,

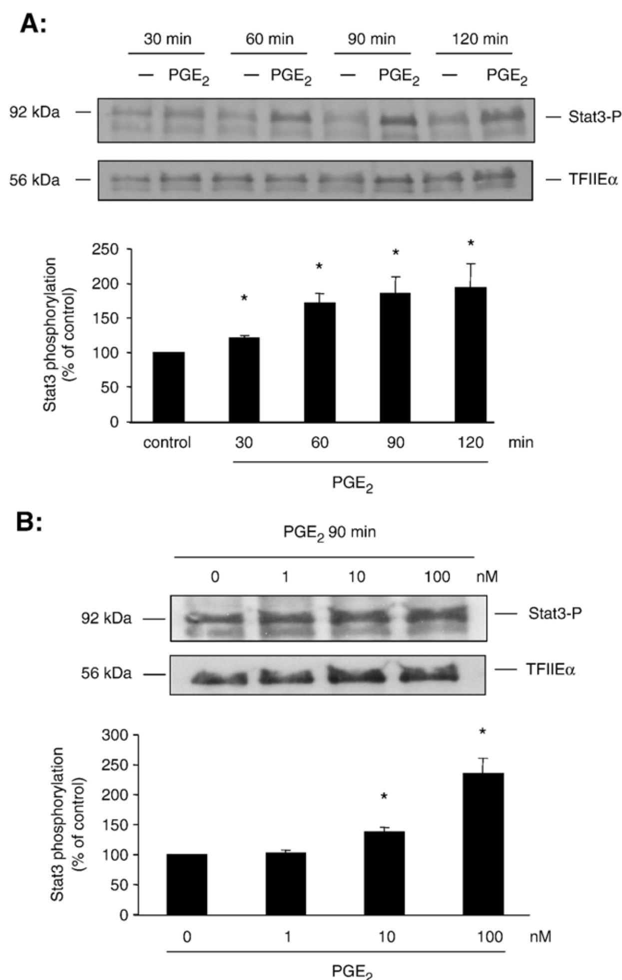

Fig. 1. PGE_2 induces a time- and concentration-dependent increase in nuclear Stat3 phosphorylation in rat ventricular cardiomyocytes. Cells were treated or not with PGE_2 (100 nM) during different time periods (30–120 min, A) or for 90 min with increasing concentrations of PGE_2 (1–100 nM, B). Thereafter, Stat3 phosphorylation (Stat3-P) was analyzed in nuclear extracts by Western blots. A representative blot is shown at the top of (A) and (B). Equal gel loading was assessed using an anti-TFIIEα antibody. Specific bands corresponding to phosphorylated Stat3 were quantified by densitometry and expressed as percentage of the corresponding control. $* = p < 0.05$ compared with control values ($n = 4$).

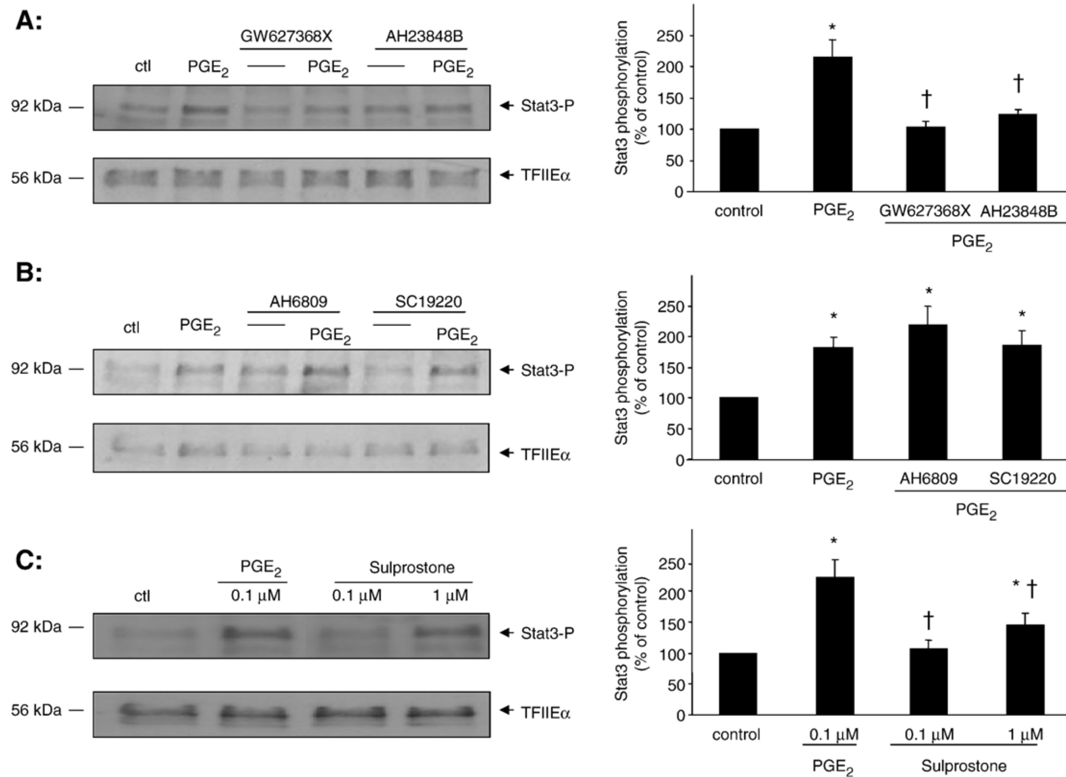

Fig. 2. PGE_2 induces an increase in Stat3 phosphorylation essentially through EP4. (A) Cells were incubated for 30 min with the specific EP4 antagonists, GW627368X (5 μM) or AH23848B (50 μM) prior to stimulation with PGE_2 (100 nM, 90 min). (B) Cells were treated for 30 min with AH6809 (EP1/EP2 antagonist, 10 μM) and SC19220 (EP1 antagonist, 10 μM) prior to stimulation with PGE_2 (100 nM, 90 min). (C) Cardiomyocytes were stimulated with the EP1/EP3 agonist sulprostone (100 nM and 1 μM) or PGE_2 (100 nM) for 90 min. Stat3 phosphorylation (Stat3-P) was analyzed in nuclear extracts by Western blotting. Representative Western blots are shown in (A–C). Equal gel loading was assessed using an anti-TFIIEα antibody. Specific bands corresponding to phosphorylated Stat3 were quantified by densitometry and expressed as percentage of the corresponding control. $* = p < 0.05$ compared with control values, $† = p < 0.05$ vs. PGE_2 ($n = 4$).

Switzerland). Thereafter, membranes were reprobed against total MEK1/2, ERK1/2 or the alpha subunit of the general transcription factor TFIIE (TFIIEα). Total MEK1/2 and ERK1/2 were used as controls for equal loading in cellular extracts, whereas TFIIEα was used as control for equal loading in nuclear extracts. Specific antibodies directed against phosphorylated Stat3, MEK1/2, ERK1/2 and total MEK1/2 were obtained from Cell Signaling Technology (Denvers, MA), while total anti-Stat3 antibody was from Upstate Biotechnology (Lake Placid, NY). Total ERK1/2, Stat1 and TFIIEα were detected by specific antibodies from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the procedure of Schreiber et al. [22]. For EMSA, oligonucleotides corresponding to the sis-inducible element (SIE) sequence localized in the promoter region of the c-*fos* gene, were annealed and radiolabeled with [γ-^{32}P]dATP. Nuclear proteins (10–20 μg) were incubated with 80’000 cpm of labeled double-stranded DNA, 1 μg of poly[d(IC)], 1 μg salmon sperm DNA and 1 μg BSA in EMSA binding buffer (20 mM Hepes pH 7.9, 5 mM $MgCl_2$, 0.5 mM EDTA, 50 mM KCl, 1 mM dithiothreitol and 6.25% glycerol) in a final volume of 25 μl. The reaction mixture was run on a 5% polyacrylamide gel at 4 °C. Gels were dried, exposed to autoradiography films and stored at −80 °C before development. In order to specify Stat3–SIE DNA binding, 0.8 μg of a polyclonal anti-Stat3 antibody (from Upstate Biotechnology or from Santa Cruz Biotechnology) or anti-Stat1 antibody (from Santa Cruz Biotechnology) were added to the reaction mixture containing the labeled probe. To confirm the specificity of the labeled SIE DNA probe, competition assay was performed using unlabeled SIE DNA sequence.

2.4. siRNA transfection

Stat3 was silenced by using a small interfering RNA (siRNA) targeting rat Stat3 with the following sequence: 5′-GGCUGAUCAUUUAUAUAAA-3′. This siRNA which was provided by Qiagen AG (Hombrechtikon, Switzerland) was annealed according to manufacturer’s instructions and then stored at −20 °C. Cardiomyocytes were transfected with siRNA in Nucleofector device (Nucleofector TM Amaxa GmBH, Cologne, Germany) by using the Nucleofector rat

cardiomyocyte kit. Briefly, 3 μg of siRNA were added to 4×10^6 cardiomyocytes suspended in 100 μl of Nucleofector rat cardiomyocyte solution. Cells were subjected to nucleofection using the cardiomyocyte specific program. For control, cells were transfected with 3 μg of non-silencing siRNA (5′-UUCUCCGAACGUGUCACGU-3′) which is ineffective in rat cells since it has no mammalian target. Transfected cells were immediately diluted in complete pre-warmed DMEM medium and seeded in 24-well plates or on fibronectin-gelatin-coated glass slides.

2.5. Microscopic analysis

After electroporation, cardiomyocytes were plated on fibronectin-gelatin-coated 22-mm glass slides. Cells were treated for 48 h with PGE_2 (1 μM) or its vehicle, 0.1% ethanol (control). For microscopic analysis of living neonatal cardiomyocytes, cells were incubated in Hepes buffer (Hepes 10 mM, KCl 5 mM, $NaHCO_3$ 5 mM, glucose 5 mM, $CaCl_2$ 1.25 mM, $MgCl_2$ 1.2 mM, NaH_2PO_4 1.2 mM, NaCl 100 mM, sucrose to 290 milliosmol/kg H_2O, pH = 7.3). To assure objectivity, all cardiomyocytes present in a microscopic field ($n \sim 50$) were analyzed. Cell surface quantification was performed using the photoshop 7.0 program.

2.6. [^{3}H]-leucine incorporation

48 h after electroporation, cardiomyocytes were incubated with PGE_2 (1 μM) or its vehicle, 0.1% ethanol (control) and co-incubated with 5 μCi/ml of [^{3}H]-leucine (Moravek Biochemicals, Brea, CA) in FCS- and leucine-free DMEM. After 12 h of stimulation, cardiomyocytes were washed twice with PBS, treated with 10% TCA and left on ice for 30 min to precipitate proteins. The precipitated proteins were washed with ice-cold ethanol 95% and then dissolved in NaOH (0.2 N). [^{3}H] incorporation was measured by scintillation counting.

2.7. Chemicals

PGE_2 and cycloheximide were purchased from Sigma–Aldrich GmbH (Buchs, Switzerland). SB203580, U0126, genistein and actinomycin D were from Calbiochem (Dietikon, Switzerland), Biomol Research Laboratories (Plymouth Meeting, PA), Alexis biochemicals (Lausen, Switzerland) and Applichem GmbH (Darmstadt, Germany), respectively. Sulprostone (EP1/EP3 agonist), SC19220 (EP1 antagonist) and AH6809 (EP1/EP2 antagonist) were obtained from Cayman (Ann Arbor, MI) and AH23848B and GW627368X (EP4 antagonists) were generously offered by GlaxoSmithKline (Brentford Middlesex, United Kingdom).

2.8. Statistical analysis

All values are expressed as mean ± SEM. Differences between groups were determined using either two-tailed unpaired Student's *t*-tests or two-way ANOVA, followed by Bonferroni's post-hoc test. $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. PGE_2 induces nuclear Stat3 phosphorylation via EP4

The activation of Stat3 by PGE_2 was first investigated by measuring Stat3 phosphorylation in the nuclear extracts of ventricular cardiomyocytes by Western blot. Since phosphorylation of Stat on tyrosine residues is necessary for its activation, we tested the effect of PGE_2 on Stat3 phosphorylation by using an antibody recognizing the tyrosine 705 phosphorylated form of Stat3. As shown in Fig. 1A, PGE_2 induces Stat3 phosphorylation on tyrosine 705 (Y705) in a time-dependent manner, reaching a maximum value after 90 min of stimulation. This phosphorylation is also

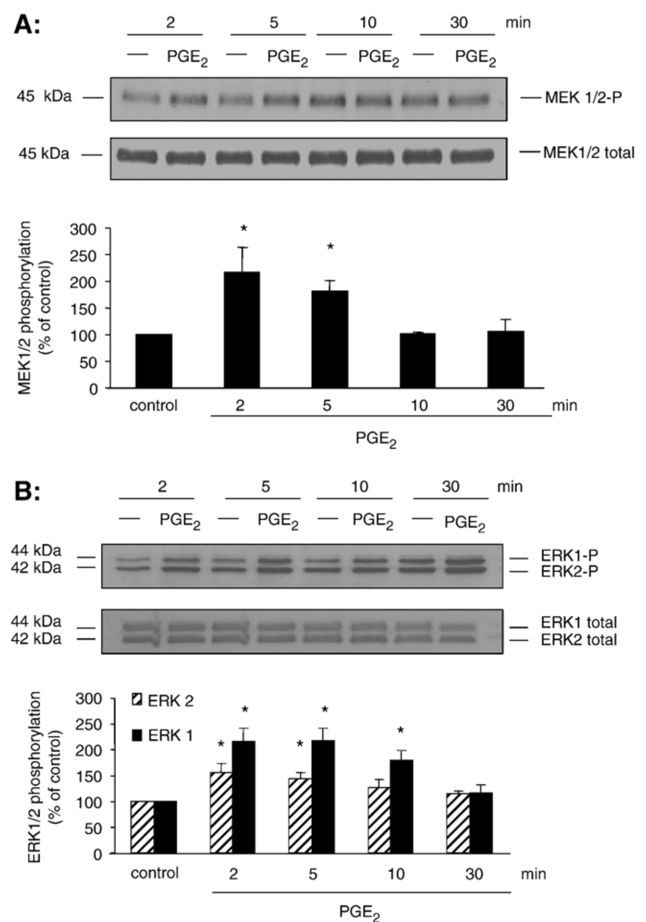

Fig. 3. PGE_2 induces a time-dependent increase in MEK1/2 and ERK1/2 phosphorylation. Cells were treated or not with PGE_2 (100 nM) during different time periods (2–30 min). Thereafter, phosphorylation of MEK1/2 (MEK1/2-P) and ERK1/2 (ERK1/2-P) was analyzed in cellular extracts by Western blots. A representative blot is shown at the top of (A) and (B). Equal gel loading was assessed using an antibody against total MEK1/2 or ERK1/2. Specific bands corresponding to phosphorylated forms of MEK1/2 (A) and ERK1/2 (B) were quantified by densitometry and expressed as percentage of the corresponding control. * = $p < 0.05$ compared with control values (n = 4–7).

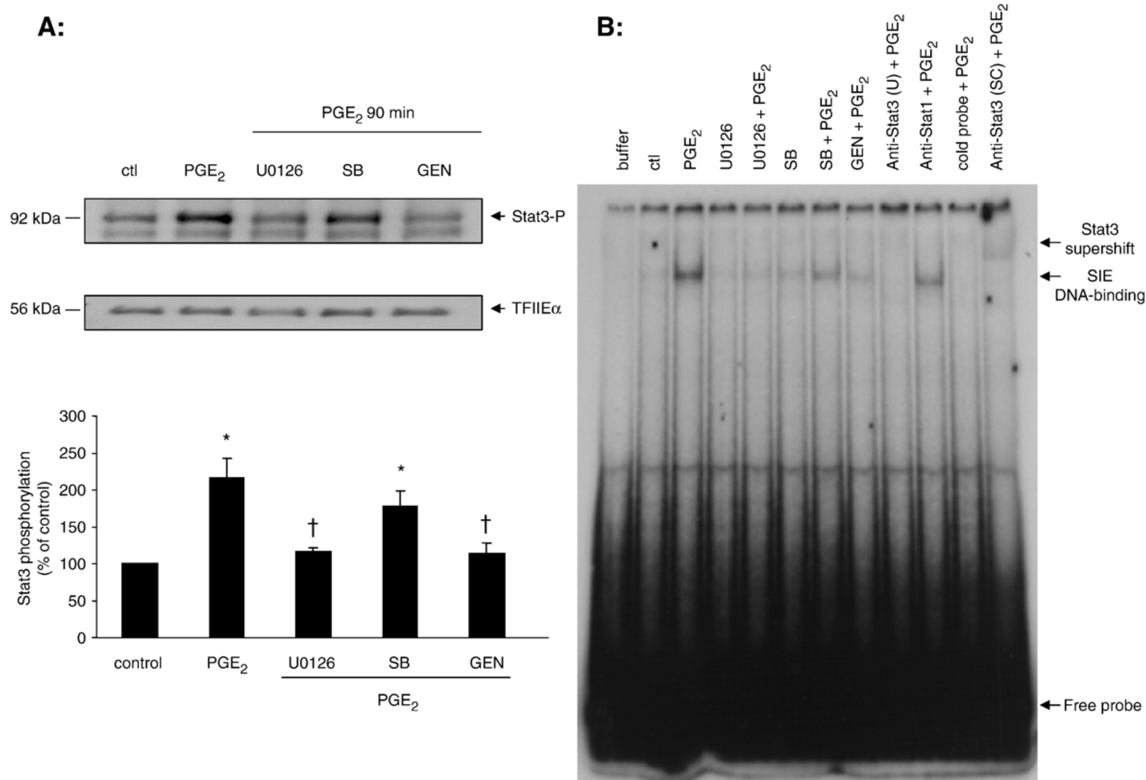

Fig. 4. Stat3 activation is abolished by U0126 and genistein but not by SB203580. Cells were incubated for 30 min with U0126 (10 μM) or SB203580 (SB, 10 μM) the inhibitors of ERK1/2 and p38 MAPK, respectively, or for 1 h with the tyrosine kinase inhibitor genistein (GEN, 100 μg/ml), prior to stimulation with PGE_2 (100 nM, 90 min). (A) Stat3 phosphorylation (Stat3-P) was analyzed in nuclear extracts by Western blotting. A representative blot is shown at the top. Equal gel loading was assessed using an anti-TFIIEα antibody and specific bands corresponding to phosphorylated Stat3 were quantified by densitometry and expressed as percentage of the corresponding control. $* = p < 0.05$ compared with control values, $\dagger = p < 0.05$ vs. PGE_2 ($n = 4–5$). (B) The DNA-binding of Stat3 was analyzed in nuclear extracts by EMSA using labeled SIE DNA. To determine Stat3 specific DNA-binding, 0.8 μg of two polyclonal antibodies against Stat3, from Upstate Biotechnology (U) and from Santa Cruz Biotechnology (SC), as well 0.8 μg of an antibody against Stat1 were used. To confirm the specificity of the labeled DNA probe, competition assay was performed using a 100-fold excess of unlabeled SIE DNA (cold probe). The figure shows a representative example from 3 experiments.

concentration-dependent, with a significant response observed after 90 min of stimulation with 10 nM of PGE_2 (Fig. 1B).

In order to determine which subtypes of the PGE_2 receptor are involved in this response we used several agonists and antagonists of the PGE_2 receptor subtypes EP1–EP4. We found that incubation of cardiomyocytes with the specific inhibitors of EP4, GW627368X and AH23848B [23,24], abolishes the PGE_2-induced phosphorylation of nuclear Stat3 (Fig. 2A). By contrast, treatment with AH6809 or SC19220, the EP1/EP2 antagonist and EP1 antagonist, respectively [25], had no effect on Stat3 phosphorylation (Fig. 2B). A weak but significant effect on Stat3 phosphorylation was observed after 90 min of stimulation with 1 μM sulprostone, the EP1/EP3 agonist [25] (Fig. 2C). Thus, our results indicate that PGE_2 induces nuclear Stat3 phosphorylation mainly via EP4, although a possible involvement of EP3 cannot be excluded.

3.2. ERK1/2, but neither p38 MAPK nor cAMP, plays a role in Stat3 activation induced by PGE_2

Since ERK1/2 and p38 MAPK have been shown to act as modulators of Stat3 activation in various cell systems [26–28], we studied their role in the activation of Stat3 induced by PGE_2 in neonatal ventricular cardiomyocytes. To this purpose we first studied the effect of PGE_2 on the phosphorylation of MEK1/2 (Fig. 3A), ERK1/2 (Fig. 3B) and p38 MAPK. As shown in Fig. 3, we found that PGE_2 induces a rapid and transient phosphorylation of MEK1/2 and ERK1/2 with significant responses observed after 2–5 min. By contrast, PGE_2 did not affect the phosphorylation level of p38 MAPK (data not shown).

To further investigate the involvement of p38 MAPK and the ERK1/2 cascade in PGE_2-induced Stat3 activation, we performed experiments using their respective inhibitors, SB203580 and U0126. SB203580 exerts its inhibitory effect by binding to the ATP site of p38 MAPK [29] while U0126 is reported to block activation of ERK1/2 by suppressing the activation of MEK1 [30,31]. Nuclear extracts were prepared after stimulation of cardiomyocytes with PGE_2 in the presence or absence of U0126 or SB203580. The phosphorylation level of Stat3 (Y705) was assessed by Western blotting (Fig. 4A) and the Stat3 DNA-binding by EMSA using the specific SIE sequence localized in the promoter region of the c-*fos* gene (Fig. 4B).

As shown in Fig. 4A, 90 min of incubation with 100 nM PGE_2 increased the phosphorylation level of Stat3. Exposure

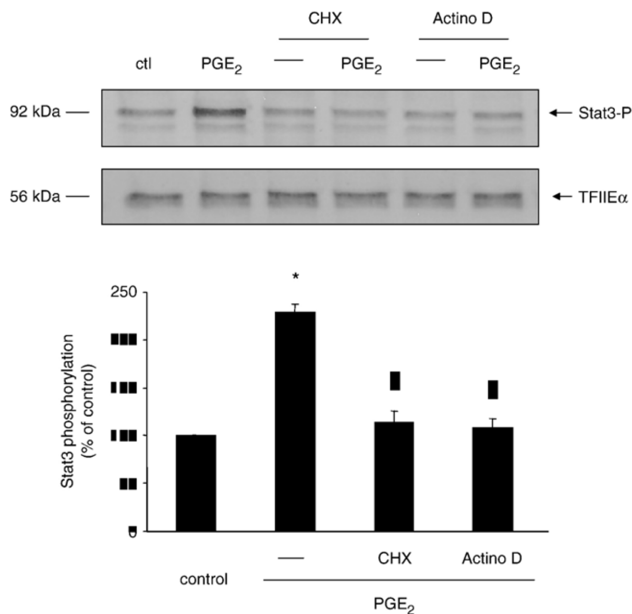

Fig. 5. Stat3 phosphorylation is abolished by cycloheximide and actinomycin D. Cells were incubated for 15 min, with the translation inhibitor cycloheximide (CHX, 10 μg/ml) or the transcription inhibitor actinomycin D (Actino D, 2.5 μg/ml) prior to stimulation with PGE_2 (100 nM, 90 min) and Stat3 phosphorylation (Stat3-P) was analyzed in nuclear extracts by Western blotting. A representative blot is shown at the top. Equal gel loading was assessed using an anti-TFIIEα antibody. Specific bands corresponding to phosphorylated Stat3 were quantified by densitometry and expressed as percentage of the corresponding control. $* = p < 0.05$ compared with control values, $\dagger = p < 0.05$ vs. PGE_2 ($n = 3$).

of cardiomyocytes to U0126 (10 μM) by blocking the ERK1/2 pathway, abolished PGE_2-induced Stat3 phosphorylation, whereas inhibiting p38 MAPK with SB203580 (10 μM), had no significant effect. As expected, the tyrosine kinase inhibitor, genistein, abolished Stat3 tyrosine phosphorylation induced by PGE_2 (Fig. 4A).

Fig. 4B illustrates that 90 min of stimulation with PGE_2 also enhanced the SIE DNA-binding of Stat3. As for Stat3 phosphorylation, PGE_2-induced Stat3 DNA-binding was completely suppressed by U0126 and genistein while it was only slightly affected by SB203580. In order to verify the specificity of the Stat3 DNA-binding, we incubated nuclear extracts from PGE_2-stimulated cardiomyocytes and labeled SIE DNA in the presence of anti-Stat3 or anti-Stat1 antibodies. In the case of anti-Stat3, we used polyclonal antibodies from two different sources, Upstate Biotechnology (U) and Santa Cruz Biotechnology (SC). As shown in Fig. 4B, the specificity of the Stat3/SIE DNA complex is demonstrated by the disappearance of this complex in the presence of anti-Stat3 (U) as well as by its shift to a higher position (supershift) in the presence of anti-Stat3 (SC). By contrast, the anti-Stat1 antibody had no effect, confirming specific Stat3 binding upon PGE_2 stimulation. During competition assay with a 100-fold excess of unlabeled SIE DNA (cold probe), the band which corresponds to the binding of Stat3 to the SIE sequence disappeared, confirming the specificity of SIE DNA binding.

Since EP4 receptor was initially characterized as coupling to the G-protein stimulating adenylate cyclase and increasing cAMP [32], we investigated the potential role of cAMP-dependent protein kinase A in PGE_2-induced Stat3 phosphorylation. Incubation of cardiomyocytes with the cell-permeant, cAMP competitive inhibitors of protein kinase A, Rp-cAMPS (10 μM) and Rp-8-CPT-cAMPS (10 μM), 30 min prior to the stimulation with PGE_2 for 90 min had no effect on Stat3 phosphorylation induced by this prostaglandin. Consistent with this observation, the cAMP-raising agent, forskolin (0.5 and 1 μM) did not increase Stat3 phosphorylation after 90 min of stimulation (data not shown).

Having observed that Stat3 activation by PGE_2 is delayed, mainly occurring after 60 min of stimulation, we hypothesized that newly synthesized proteins are necessary for this response. Therefore, we blocked translation and transcription with cycloheximide and actinomycin D, respectively. As illustrated in Fig. 5, both inhibitors abolished nuclear Stat3 phosphorylation induced by PGE_2 without affecting Stat3 expression in cellular extracts (data not shown). Our results indicate a role of ERK1/2 but not of p38 MAPK in the activation of Stat3 induced by PGE_2. Moreover, it appears that newly synthesized protein(s) are necessary for this response.

3.3. Stat3 plays a role in PGE_2-induced hypertrophy

PGE_2 was shown to increase protein synthesis and cell surface in cardiomyocytes [5]. In order to evaluate whether

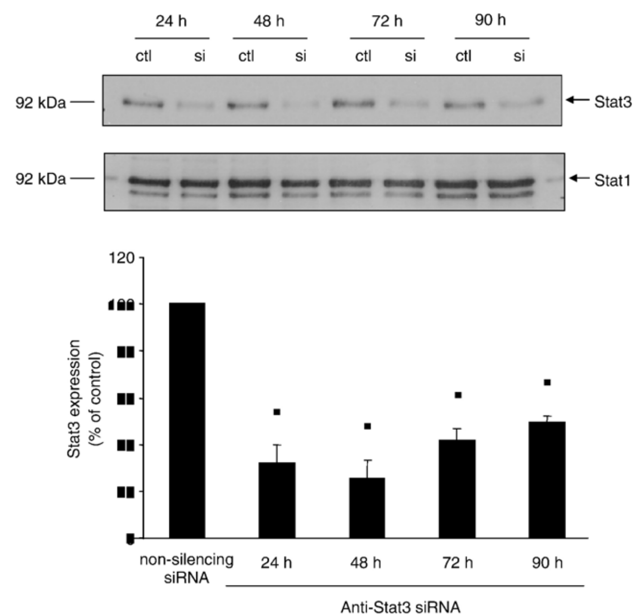

Fig. 6. Stat3 expression is strongly reduced by siRNA targeting Stat3. Cardiomyocytes were electroporated with specific siRNA targeting Stat3 (si) or non-silencing siRNA (control, ctl) for different periods of time, and Stat3 expression in cellular extracts (15 μg) was analyzed by Western blotting. A representative blot is shown at the top. Equal gel loading and specificity were assessed using an anti-Stat1 antibody. Specific bands corresponding to Stat3 were quantified by densitometry and expressed as percentage of the corresponding control. $* = p < 0.05$ compared with control values ($n = 3$).

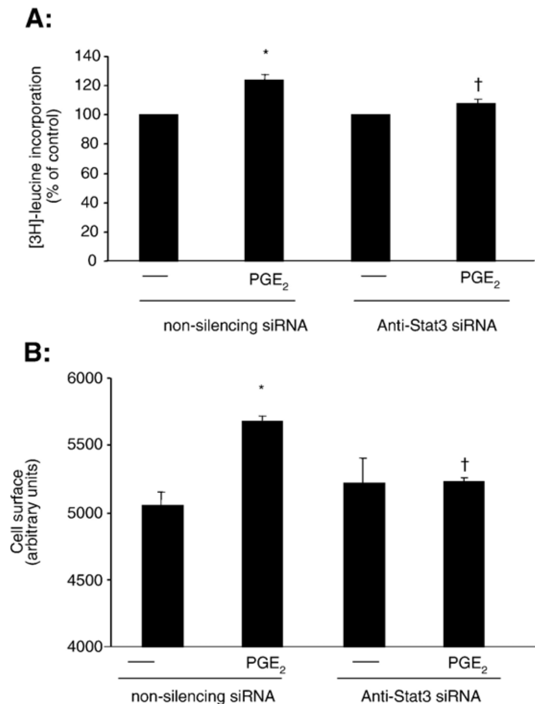

Fig. 7. siRNA targeting Stat3 blocks PGE_2-induced increase in cell size and protein synthesis. 40 h after electroporation, cardiomyocytes were treated or not with PGE_2 (1 μM). [^{3}H]-leucine incorporation into total protein was measured after 12 h of incubation with PGE_2 (A). Microscopic analysis was performed after 48 h of exposure to PGE_2 and cell surface (300–360 cells/condition) was determined (B). $* = p < 0.05$ compared with control values, $\dagger = p < 0.05$ vs. PGE_2 ($n = 3$–4).

Stat3 plays a role in these PGE_2-induced hypertrophic responses, we silenced Stat3 by using small interfering RNA (siRNA) targeting Stat3. As shown in Fig. 6, Stat3 protein expression was decreased by 68±8% and 74±7%, 24 h and 48 h, respectively, after transfection of cardiomyocytes with siRNA directed against Stat3, when compared to cells transfected with non-silencing siRNA. This Stat3 knock-down effect remained sustained until 90 h after transfection with anti-Stat3 siRNA (Fig. 6). By contrast, Stat1 expression was not affected in these Stat3-silenced cells, confirming the specificity of the siRNA used.

As illustrated in Fig. 7, in cells transfected with non-silencing siRNA, PGE_2 induced a significant increase in both, protein synthesis, as measured by [^{3}H]-leucine incorporation into total protein (Fig. 7A), and cell surface (Fig. 7B). These responses were abolished when cells were transfected with anti-Stat3 siRNA. These findings indicate that Stat3 plays a key role in PGE_2-induced cardiomyocyte hypertrophy.

4. Discussion

The major finding of this study is that in neonatal ventricular cardiomyocytes, PGE_2 induces the activation of Stat3 which plays an essential role in PGE_2-induced increase in protein synthesis and cell size.

In the present work, we show for the first time that in ventricular cardiomyocytes, PGE_2 induces Stat3 activation characterized by an increase in its tyrosine phosphorylation level and its DNA-binding activity. Using various agonists and antagonists of the G-protein-coupled PGE_2 receptor subtypes EP1–EP4, including two potent and specific inhibitors of EP4 [23,24], we found that PGE_2 activates Stat3 mainly through the EP4 receptor although we cannot exclude a possible involvement of EP3. Consistent with these results, it has recently been shown that PGE_2 induces hypertrophic responses in cardiomyocytes acting through the EP4 receptor subtype [5,6]. Moreover several studies reported a high expression level of EP4 in heart tissue [33–35].

Interestingly, the selective, cell-permeant, cAMP competitive inhibitors of protein kinase A, Rp-cAMPS and Rp-8-CPT-cAMPS had no effect on PGE_2-induced Stat3 phosphorylation. Consistently, the adenylate cyclase activator forskolin did not induce Stat3 phosphorylation. These results are in agreement with recent investigations on EP4 signaling. Indeed, although initial studies on EP4 indicated that activation of this receptor increases cAMP formation [32], it has been shown recently that PGE_2 stimulation induces the expression of early growth response factor-1 mainly through ERK1/2 [36].

In our study we also found a role for ERK1/2 in PGE_2 signaling. Indeed, we observed that in ventricular cardiomyocytes, 100 nM PGE_2 induces an important transient

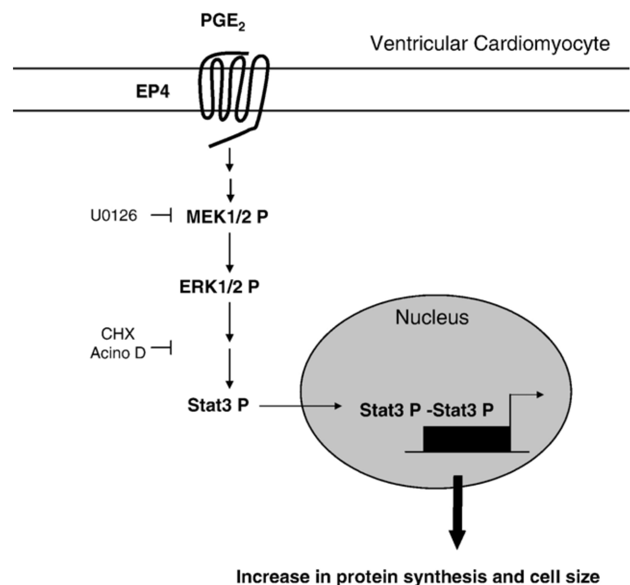

Fig. 8. Proposed model of PGE_2-induced Stat3 activation and hypertrophic responses in the neonatal ventricular cardiomyocyte. PGE_2 activates the EP4 receptor subtype which induces activation of the MEK1/2–ERK1/2 signaling cascade leading to the phosphorylation of Stat3 which also needs newly synthesized protein(s). After its phosphorylation, Stat3 dimerizes and translocates to the nucleus where it promotes the transcription of target genes leading to an increase in protein synthesis and cell size. Abbreviations: U0126, MEK1/2 blocker; CHX (cycloheximide), translation inhibitor and Actino D (actinomycin D), transcription inhibitor.

increase in MEK and ERK1/2 phosphorylation reaching maximal values after 2–5 min. By contrast, PGE_2 appears to have no effect on p38 MAPK. These results are in agreement with those of Mendez and LaPointe [5] who recently reported that 1 μM PGE_2 induces the phosphorylation of ERK1/2 through the EP4 receptor. We also found that inhibition of the serine/threonine kinase ERK1/2, but not of the p38 MAPK pathway, abolished not only PGE_2-induced Stat3 DNA-binding but also its phosphorylation on tyrosine.

On the one hand, these results clearly demonstrate that ERK1/2 activation is a necessary event for PGE_2-induced Stat3 activation. On the other hand, they further indicate that Stat3 is not the direct target of ERK1/2 in our system, although it has been shown *in vitro* that Stat3 is an excellent substrate for ERKs [37] and that activation of Stat3 can be modulated by serine phosphorylation [26,37]. The indirect role of ERK1/2 is further supported by our finding that the translation inhibitor cycloheximide blocks the PGE_2-induced Stat3 tyrosine phosphorylation. Our observations are consistent with that of Ng et al. [27], showing that in cardiomyocytes, interleukin-1β-induced Stat3 tyrosine phosphorylation is reduced following ERK1/2 inhibition and requires protein synthesis. Taking into account both, the fact that linking PGE_2-induced ERK1/2 activation and Stat3 phosphorylation requires an intermediate tyrosine kinase and our finding that protein synthesis is involved in the delayed phosphorylation of Stat3, we propose two hypothesis. First, newly synthesized protein(s) would remain in the cell and being or inducing a non-receptor tyrosine kinase would lead to Stat3 activation. Second, newly synthesized protein(s) would be released and would act in an autocrine manner to stimulate a receptor-associated tyrosine kinase. This more indirect mechanism could involve cytokines of the interleukin-6 (IL-6) family whose receptors are expressed in cardiomyocytes [38] and are well known to activate Stat3. Consistent with this hypothesis, angiotensin II was shown to induce Stat3 tyrosine phosphorylation through the autocrine action of secreted IL-6 family cytokines [13]. Moreover, we found previously that in neonatal rat ventricular cardiomyocytes, PGE_2 induces IL-6 mRNA expression [2]. Further investigations are needed to elucidate all the steps involved in the delayed activation of Stat3 by PGE_2.

An important part of our study was to investigate the role of Stat3 in PGE_2-induced cardiomyocyte hypertrophy. To this purpose we determined protein synthesis and cell size, two key features of cardiomyocyte hypertrophy which have been shown to be increased following stimulation with PGE_2 [5]. By electroporating neonatal ventricular cardiomyocytes with siRNA targeting specifically rat Stat3, we obtained an approximately 70% inhibition of Stat3 expression. In these Stat3 silenced cardiomyocytes, the PGE_2-induced increase in protein synthesis and cell size was abolished. These results demonstrate that Stat3 activation is essential for PGE_2-induced hypertrophic responses in ventricular cardiomyocytes, a finding which is consistent with the present knowledge of Stat3 playing an important role in the regulation of cardiac hypertrophy [7–9,14,15].

Taking into account all our data, we propose the model shown in Fig. 8. In neonatal ventricular cardiomyocytes, PGE_2 activates the EP4 receptor which leads to the stimulation of the MEK1/2–ERK1/2 pathway inducing the phophorylation of Stat3. According to our experiments with actinomycin D and cycloheximide, the PGE_2-induced Stat3 phosphorylation involves newly synthesized yet-to-be-determined protein(s). After its phosphorylation, Stat3 dimerizes and translocates to the nucleus where it induces the transcription of target genes leading finally to characteristic hypertrophic responses such as an increase in protein synthesis and cardiomyocyte size.

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